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HETEROXANTHIN, DIATOXANTHIN AND DIADINOXANTHIN FROM *TRIBONEMA AEQUALE**

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Abstract—The principal crystallizable xanthophylls of *Tribonema aequale* are heteroxanthin, diatoxanthin, and diadinoxanthin. The latter pigment from *Tribonema* is identical with the diadinoxanthin from *Euglena* and the diatom *Nitzschia*. Description and identification of the pigments have been based upon comparative i.r., NMR and mass spectra.

INTRODUCTION

MOST YELLOW-GREEN algae, Heterokontae, contain three principal xanthophylls[1,2] sometimes accompanied by a xanthophyll partial ester.[1,3,4] Disagreement concerning the properties and identification of the three principal xanthophylls may be attributed to variations in the organisms used, the homogeneity of the cultures, the chromatographic methods for isolation and comparison of the individual pigments, and the physical and chemical methods for characterization of the pigments.[1-5]

From precision mass spectrometry, the empirical formulae of the three major xanthophylls isolated from a natural, badly contaminated stand of *Vaucheria* spp. were $C_{40}H_{56}O_4$ (heteroxanthin), $C_{40}H_{54}O_2$ (isomeric with acetylenic dihydroxyxanthophylls) and $C_{40}H_{54}O_3$ (identical or isomeric with diadinoxanthin).[1] From comparative chromatography, the principal xanthophylls from homogeneous cultures of several species of *Vaucheria* were reported to be diatoxanthin (or zeaxanthin in one species) and diadinoxanthin.[4] In some instances, the wavelengths of the absorption maxima of the diatoxanthin were shorter than those reported for diatoxanthin from diatoms and from cryptomonads.[1,2,4]

We have now isolated the principal xanthophylls from pure cultures of *Tribonema aequale* by chromatography, rechromatography and crystallization. Their mol. wts., spectral properties and chromatographic behavior were then determined and compared with those of corresponding preparations from two species of *Vaucheria* and from *Nitzschia closterium* var. *minutissima*. The NMR values of all these preparations were compared with those of carotenoids of established structure.

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RESULTS

Culture of Tribonema

Tribonema aequale was cultured in a medium[6] containing 0·1 g KNO_3, 0·05 g KH_2PO_4, 0·05 g $MgSO_4 \cdot 7H_2O$, 1·0 g glucose, 1·0 g yeast extract, and 250 ml soil extract made to 1 l. with nonchlorinated, well water. In this medium, cellular growth exceeded that in a similar medium which lacked the soil extract. Organisms grown in both media provided the same xanthophylls.

The culture medium, 2 l. in each of 8–16, low-form, culture flasks, was autoclaved (17 lb, 40 min), cooled, inoculated under sterile conditions, and placed under fluorescent lights before a north window for 10–14 days. The algal filaments were collected by pouring the culture medium through a 14-mesh, wire screen.

Extraction of Pigments

The moist alga was added to about 2 l. boiling water containing 3 g Na_2HPO_4 for 3 min, then cooled with ice. The alga, again collected on the screen, was pressed lightly to remove water, and extracted with several portions of methanol and of ether until all the pigments were removed. The air-dried, extracted cells from each 2 l. portion of the culture weighted about 1 g.

The combined, green extracts were filtered through a loose pad of cotton. Then 500-ml portions of the filtrate were diluted with 1 l. of ether and extracted at least twice with 1 l. portions of brine. The combined ether solutions were evaporated, and the pigment residue was stored under vacuum and refrigeration at –20°.

Heteroxanthin

The pigment residue, from the *Tribonema* grown in 16 flasks, was dissolved in benzene, adsorbed in eight to ten sugar columns (*ca.* 8 × 36 cm), and washed with more benzene. This development provided a very strongly sorbed, yellow zone containing the heteroxanthin. This zone was removed from all the columns and packed into fresh tubes. The pigment was eluted with ethanol plus ether, which was washed with water and evaporated under vacuum. The residual yellow pigment was dissolved in ether–light petroleum (1:2) and readsorbed in three, large, sugar columns, which were washed with light petroleum containing 0·5% *n*-propanol. After elution and recovery, as described above, the heteroxanthin was dissolved in ether–light petroleum (1:1) and adsorbed on sugar in a large column. When washed with ether–light petroleum (7:3), it separated into two zones. The pigment from the minor, upper zone could not be induced to crystallize. The heteroxanthin from the major lower zone was crystallized from benzene by the addition of light petroleum.

This crystalline pigment (about 8 mg from some 170 cultures) melted at 178–180° μc. Absorption maxima of an ethanol solution were at 448 and 478 nm, and the shape of the curve was like that of diadinoxanthin (without any indication of *cis*-peaks). With HCl in ethanol, the heteroxanthin was unaltered as indicated by the unchanged, spectral absorption curve. The chromatographic behavior of heteroxanthin from *Tribonema* was identical with this pigment from *Vaucheria* (cellulose TLC with light petroleum plus 3% *n*-propanol, also with light petroleum and ether (1:1) plus 0·75 and 1·0% *n*-propanol, and with various solvent mixtures). With sugar columns and with TLC, the heteroxanthin was much more sorbed than neoxanthin. The absorption maxima in the i.r. (KBr pellet) will be reported in "Chemical Communications."

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When dissolved in C_5D_5N, the heteroxanthin exhibited strong resonance in the NMR (τ 8·80–8·88) indicative of "impurities" or hydrocarbon residues. These impurities were removed by recrystallization from the C_5D_5N (0·3 ml) by dropwise addition of "Lipopure Hexane" (Applied Science Laboratories, Inc., State College, Pennsylvania, U.S.A.) 12 ml, by recrystallization from $CHCl_3$ (0·3 ml) by the addition of the Lipopure Hexane, and by drying in a vacuum. This product exhibited NMR resonances at τ 8·06, 8·15, 8·43, 8·51, 8·79 and 8·87 in C_5D_5N and at τ (8·05, 8·08, 8·12), 8·79, (8·85, 8·87, 8·90) and 9·18 in $CDCl_3$ (Ref. HMS).

This system of resonances does not correspond to those reported for any known carotenoid. Some of the resonances correspond to those of methyl groups in the half of carotenoid molecules with terminal 3-hydroxy β-rings adjoining —C≡C— units. For example, the average of τ values (C_5D_5N, Ref. HMS) and the position of the methyl groups (at carbon atoms, 1, 5, 9 and 13) for diadinochrome, diadinoxanthin (ex. *Euglena*, *Nitzschia* and *Tribonema*), and diatoxanthin (ex. *Tribonema*) were: 1, 8·87, 8·79; 5, 8·14; 9, 8·04; 13, 8·14.

The mass spectrum indicated that the empirical formula was $C_{40}H_{56}O_4$. The fragmentation pattern was like that observed with heteroxanthin from *Vaucheria* (unpublished).[1]

All these properties establish the identity of heteroxanthin from *Tribonema* and from *Vaucheria*. Additional investigations, still in progress, indicate heteroxanthin to be a tetrahydroxy acetylenic carotenoid, probably 3,5,6,3′-tetrahydroxy-5,6-didhydro-7′,8′-didehydro-β-carotene.

Diatoxanthin

The pigments extracted from *Tribonema* (20 culture flasks) were dissolved in benzene, adsorbed in 10 sugar columns and washed with benzene. Large, contiguous, yellow zones were removed from the center of the column, and the pigments were eluted and recovered. Dissolved in ether–light petroleum (1:1), this pigment mixture was readsorbed on sugar and washed with light petroleum plus 0·5% *n*-propanol. The xanthophyll mixture, from which most of the chlorophylls had been separated, was again recovered. Readsorbed on sugar from solution in ether–light petroleum (2:3), it provided indication of four, large, contiguous zones occupying the upper, central section of the column. These were recovered together, readsorbed on sugar from ether–light petroleum, and washed with light petroleum–7·5% acetone. This provided a moderately adsorbed light-orange zone followed by three, light-yellow zones. The xanthophyll from the lowest, orange zone was recovered, dissolved in ether and adsorbed in a column (5 × 28 cm) of magnesia (formerly Micron Brand 2641,[2] now Sea Sorb 43 from Fisher Scientific Company) plus Clite 545 (1:1). Washed with ether plus increasing concentrations (5–30%) of *n*-propanol, this pigment formed a principal, well-defined orange zone midway through the magnesia column. The xanthophyll recovered from this zone was recrystallized from benzene by the addition of light petroleum. Yield *ca.* 7 mg.

This crystalline pigment melted at 182–186° μc. Mass spectra indicated the formula $C_{40}H_{54}O_2$. Principal fragmentation products were the same as those observed with diatoxanthin from *Vaucheria* (unpublished)[1] and corresponded to those required for the structure proposed for this pigment.[5] Absorption maxima in ethanol, 453 and 483 nm, were not altered by HCl. Absorption maxima in i.r. (KBr pellet) were: 3417, 2960, 1469, 1379, 1060, 975, corresponding closely to the expected values.

The diatoxanthin could be identified by comparative NMR values even though resonance data for this pigment had not been published and authentic reference material was not available. As noted above, some of the NMR values for diatoxanthin were identical with those for half molecules containing terminal 3-hydroxy β-rings adjoining —C≡C— units. The remaining resonances (C_5D_5N, Ref. HMS) corresponded to the average values for the methyl groups in half molecules containing terminal 3-hydroxy β-rings. For cryptoxanthin, zeaxanthin and lutein, the location of the methyl groups and the τ values were: 1, 8·97, 8·98; 5, 8·33; 9, 8·12; 13, 8·10. Those for diatoxanthin were: 1, 8·96, 8·96; 5, 8·31; 9, 8·15; 13, 8·09.

All of these results establish the chemical identity of the $C_{40}H_{54}O_2$ pigment from *Tribonema* as diatoxanthin. Part of the earlier difficulty in preparing the larger quantities of this xanthophyll in a state of high purity may be attributed to the decrease in the selectivity of the heavily loaded, chromatographic columns, especially the sugar columns.[7]

Diadinoxanthin

The pigment mixture extracted from *Tribonema* (30 cultures) was dissolved in 15–20 ml ether which was then diluted to 300 ml with light petroleum. Aliquot portions were adsorbed in three large sugar columns and washed with ether–light petroleum (3:7). Several contiguous yellow zones soon separated below the green chlorophyll zone. These were removed together, and the pigment was recovered. Five such preparations were combined, dissolved in ether–5% *n*-propanol, adsorbed on magnesia (Sea Sorb 43) plus Celite (1:1) in a column (8 × 36·5 cm). Washed with ether plus 20–70% *n*-propanol, the mixture provided a lower, dense, light-yellow, xanthophyll zone, which moved more than half-way through the column preceding a heavy yellow zone. The pigment recovered from the lower yellow zone was crystallized from benzene by the addition of light petroleum. Yield from *ca.* 170 cultures 10 mg.

Crystals of this pigment melted at 159–162° μc. Mass spectra indicated a formula of $C_{40}H_{54}O_3$. The principal fragmentation products were identical with those observed with diadinoxanthin from *Euglena* and from *Nitzschia*.[8] The absorption maxia in ethanol, 448 and 478 nm, were shifted to 431 and 459 nm by HCl, indicating a 5,6-epoxy group, also in agreement with the properties of diadinoxanthin. The principal absorption peaks in the i.r. (KBr pellet) were identical with those for diadinoxanthin from *Euglena* and from *Nitzschia*.[8]

The NMR τ values (C_5D_5N, Ref. HMS) for diadinoxanthin from *Tribonema* corresponded with those for this pigment from *Euglena* and *Vaucheria*. Some of these values corresponded to those of the methyl groups in 3-hydroxy β-rings adjoining —C≡C— groups as described above for heteroxanthin. Others agreed with the average τ values for molecules with terminal 3-hydroxy 5,6-epoxy rings. For violaxanthin, luteoxanthin and neoxanthin, these values with the location of the methyl groups were: 1, 9·02; 8·90; 5, 8·89; 9, 8·15; 13, 8·13. For diadinoxanthin from *Tribonema*, *Euglena* and *Nitzschia*, the average values with their assignments were: 1, 9·01, 8·87; 5, 8·87; 9, 8·14; 13, 8·14. These results establish the chemical identity of diadinoxanthin from *Tribonema aequale*, *Vaucheria* and *Euglena*.

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Heteroxanthin and Diadinoxanthin from Vaucheria spp.

An unidentified species of *Vaucheria* (*ca.* 260 g) was collected in North Carolina and shipped via air mail. It was washed and scalded, and the pigments were extracted with methanol plus light petroleum as described before.[1] The principal pigments, separated by chromatography in ten sugar columns, proved to be heteroxanthin and diadinoxanthin. These two pigments were identical with the corresponding pigments previously isolated from another species of *Vaucheria*[1] and also from the *Tribonema*. The corresponding pigments exhibited identical i.r., NMR, mass spectra, electronic spectra, chromatographic behavior and reactions with HCl in ethanol.

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